REMARKS

Applicants acknowledge with appreciation receipt of the Notice of Allowance mailed August 11, 2003. By this Amendment, Claim 36 is being amended, and no claims are being added or canceled. Therefore, Claims 30-31, 35-40 and 44-47 are pending in the application.

This Amendment is being submitted concurrently with a Request for Continued Examination ("RCE"), an Information Disclosure Statement ("IDS"), a Substitute Form PTO-1449A and copies of the listed references and/or information. The IDS is intended to make of record various papers that have arisen in the course of legal proceedings in Japan and Europe.

The Amendments of the Claims

Claim 36 has been amended to correct an obvious error in antecedent basis. As amended, Claim 36 recites an instrument that includes a sealed transmission path between the reaction vessel and the detector. Support for amendment is found in the specification at, for example, page 14, lines 24-34; page 28, line 34 through page 29, line 9; and in FIG. 7, which illustrates a fiber optic embodiment of a sealed transmission path. Accordingly, no new matter is introduced by virtue of the amendment.

Concurrently Filed IDS

The enclosed IDS discusses the origins of various references that have been cited in legal proceedings involving counterpart Japanese or European patents. Three particular references are addressed further below.

European Patent application EP 0 487 218 A2 (Cite No. 25) published on May 27, 1992, and therefore does not qualify as prior art against the instant application, which has an effective filing date of May 2, 1991. Furthermore, the corresponding U.S. application Serial No. 07/784,888 was involved in an interference with parent application Serial No. 07/695,201, now U.S. Patent No. 5,994,056 (Interference No. 103489), in which the parent '201 application was awarded priority.

Cite No. 41 corresponds to the cover page, title page and pages i-iii, 1, 2, 48-56 and 65 of Eigen et al., "Report on Evolution Research," Department of Biochemical Kinetics, Max Planck Institute für Biophysikalische Chemie ("Eigen Report"), which were brought to the attention of the Japanese Patent Office in a Japanese counterpart of the instant application (Japanese Patent

No. 3136129). Cite No. 41 contains pages of the Eigen Report in addition to those that have already been made of record in the instant application in a Supplemental Information Disclosure Statement filed July 19, 2001 (Cite No. 19).

This reference was purportedly made publicly available April 18-20, 1991 at a meeting or conference at the Max Planck Institute in Göttingen, Germany, according to declarations submitted during prosecution of European Patent EP 0 583 265 (see English translations of declarations by M. Eigen, R. Winkler-Oswatitsch, B. F. Lindemann, A. Schwienhorst and R. Günther, Cite No. 42). However, this reference cannot qualify as prior art, because its alleged publication date was less than one year before the effective filing date of the present application, and because the presently claimed invention was actually reduced to practice prior to the alleged publication date.

As evidenced by the enclosed Declaration of inventor Russell G. Higuchi Under 37 CFR § 1.131 ("Higuchi Declaration;" attached hereto as Exhibit 1), an embodiment of the instantly claimed instrument was actually reduced to practice before April 15, 1991, which is before the alleged publication date of April 18-20, 1991 of Cite No. 41. As is apparent from ¶5 of the Higuchi Declaration, an instrument in accordance with the present claims was used to monitor a polymerase chain amplification reaction in real time. The specific instrument used included a thermal cycler adapted to receive at least one reaction vessel and a detector operable to detect, by way of an optical fiber, a fluorescence signal while the amplification was in progress and without having to open the reaction vessel. The reaction vessel in which the polymerase chain amplification reaction was carried out contained an amplification mixture comprising a target nucleic acid, reagents for nucleic acid amplification and a detectable nucleic acid binding agent (ethidium bromide). The trace of the results of the experiment (attached as Exhibit A to the Higuchi Declaration), which Dr. Higuchi received and understood before April 15, 1991, demonstrates that the instrument worked for its intended purpose.

Applicant notes that while it has opted to antedate the alleged publication date of Cite No. 41, Applicant has in no way admitted or conceded that: (1) Cite No. 41 anticipates and/or obviates the Claims; (2) that Cite No. 41 constitutes a printed publication; and/or (3) that, if Cite No. 41 constitutes a printed publication, it published prior to the effective filing date of the instant application. Applicant expressly reserves the right to address any or all of these issues at a later date.

Cite No. 47 is an Annex (Annex 2) reproduced from Cite No. 46 (opposition brief submitted by Eppendorf). The Annex purports to describe an experiment performed using a system that allegedly corresponds to a measuring station Eppendorf has sold since the1970's, called a Measuring Station 5086 (described in Cite No. 30). However, it is evident that significant modification to Measuring Station 5086 was required for the experiment to be carried out.

The system used for the experiment, illustrated in FIG. 1 of Annex 2, included an Eppendorf Photometer 1101 with fluorescence attachment 1030, a mercury lamp and two thermostatted water baths (Eppendorf thermostats 2762 and 2763), filled with 1.2 and 3 liters of water, respectively, tempered to 96°C and 5°C, respectively. The water baths were connected to the tempering device of the fluorescence attachment by silicone tubing (see Cite No. 47 at page 2).[1] The system also included an analog recorder (Cite No. 47 at page 2, ¶5).

This system was used to monitor a PCR reaction carried out in the presence of SYBR® Green fluorescent dye (Cite No. 47 at Section 3.2). However, this experiment did not utilize the Measuring Station 5086 as originally disclosed. In particular, the two-bath system used in the experiment in Cite No. 47 does not correspond to the single-bath Measuring Station 5086 (see Cite No. 30) as alleged (see Cite No. 47 at page 2, ¶1). Cite No. 47 also alleges that the experiment could have been carried out with a single water bath instead of two water baths, but that this would have "taken longer" (see Cite No. 47 at page 2, ¶4). However, such an assertion is both misleading and irrelevant since the actual Measuring Station 5086 (as advertised and sold commercially) did not include a thermocycler at the time of the present invention. Accordingly, Cite No. 47 is without merit.

Conclusion

It is respectfully submitted that Claims 30-31, 35-40 and 44-47 satisfy all of the criteria for patentability and are in condition for allowance. An early indication of the same is therefore kindly requested.

[1] All references to Cite No. 47 are to the English translation except as to Figures.

No fees beyond those included with the enclosed RCE transmittal are believe to be due in connection with this Amendment. However, the Patent Office is authorized to take any required fees from Applied Biosystems Deposit Account No. **01-2213 (Order No. 4380US)**.

Respectfully submitted,

Date: Oct. 23, 2003

Vincent M. Powers
Attorney for Applicant
Reg. No. 36,246
Telephone: 650-638-6492

CORRESPONDENCE ADDRESS

Customer Number 22896
Applied Biosystems
850 Lincoln Centre Drive
Foster City, California 94404
TEL: 650-570-6667
FAX: 650-638-6677

Case No. 4380US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Russell G. Higuchi**	)	Group Art Unit: 1743
Serial No.: 08/968,208	)	Examiner: J. Snay
Filed: November 11, 1997	)	Confirmation Number:
For: INSTRUMENT FOR MONITORING NUCLEIC ACID AMPLIFICATION	)	

DECLARATION OF RUSSELL G. HIGUCHI, Ph.D. UNDER 37 CFR § 1.131

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, RUSSELL G. HIGUCHI, Ph.D., declare as follows:

1. I am the inventor of the above-captioned patent application and am familiar with its contents and currently pending claims.

2. I received a Doctor of Philosophy degree in Molecular Biology at the University of California, Los Angeles in 1980.

3. At the time I made the instrument claimed in the above-captioned patent application, I was a Scientist at Cetus Corporation.

4. Prior to April 15, 1991, the invention claimed in the above-captioned application was actually reduced to practice in an experiment carried out to confirm that my proposal to monitor PCR reactions in real time was possible, and the results of the experiment were faxed to me. I

CMD
RNS Law Dept.

placed a copy of the experiment results that were faxed to me into my notebook on or before April 15, 1991.

5. In the experiment, amplification of Y-chromosome-specific repeat sequences by a polymerase chain reaction (PCR) carried out in the presence of ethidium bromide ("EtBr") was monitored in real time using an embodiment of an instrument claimed in the captioned application. The PCR reaction (100 µl in a 0.5 ml polypropylene centrifuge tube with its cap removed) contained: 10 mM Tris-HCl, pH 8.3; 4 mM $MgCl_2$; 2.5 units *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT); male DNA-specific primers (15 pmole each primer); 20 ng human male target DNA; and 0.5 µg/ml EtBr. The reaction was overlayed with mineral oil (2 drops) to prevent evaporation. Continuous monitoring of the PCR in progress was accomplished using a Fluorolog-2 fluorometer (SPEX, Edison, NJ) equipped with a fiber optic accessory (SPEX cat. no. 1950) to both send excitation light to, and receive emitted light from, the PCR tube. The probe end of the fiber optic cable was attached with "5-minute-epoxy" to the open top of the PCR tube, effectively sealing it. The exposed top of the PCR tube and the end of the fiber optic cable were shielded from room light and the room lights were kept dimmed during the PCR amplification. Thermocycling and fluorescence measurement were started simultaneously. Thermocycling proceeded for 30 cycles at 94 °C for 1 min. and 50 °C for 1 min. in a model 480 thermocycler (Perkin-Elmer Cetus) using a "step-cycle" program. Excitation was at the 500 nm band with about 2 nm bandwidth with a GG 435 nm cut-off filter (Melles Grist, Inc., Irvine, CA) to exclude second-order light. Emitted light was detected at 570 nm with a bandwidth of about 7 nm. An OG 530 nm cut-off filter was used to remove the excitation light. A time-base scan with a 10 second integration time was used and the emission signal was ratioed to the excitation signal to control for changes in light-source intensity. Data were collected using the dm3000f, version 2.5 (SPEX) data system.

6. A copy of the results of the experiment, which I received and understood prior to April 15, 1991, is attached hereto as EXHIBIT A. The fluorescence trace as a function of time clearly shows amplification of the target DNA. Fluorescence intensity is minimum at the denaturation temperature (94 °C) and maximum at the annealing/extension temperature (50 °C). The fluorescence maxima at the annealing/extension temperature begins to increase at about 4000 seconds of thermocycling, and continues to increase with time, indicating that increasing

CMO
RAS Law Dept.

amounts of double-stranded DNA amplification product are being produced as a function of time. The fluorescence minima at the denaturation temperature do not significantly increase, presumably because at this temperature there is no double-stranded DNA for EtBr to bind.

7. On the fluorescence trace as faxed to me, it is written that the annealing/extension temperature is 55 °C. The correct annealing/extension temperature of the experiment, as indicated above, is 50 °C. When I placed a copy of the experiment results that were faxed to me into my notebook, I corrected the temperature that was written on the trace based on information I received after the experiment was conducted. In any event, the results of the experiment would be the same whether the annealing/extension temperature had been 50 or 55 degrees as both are appropriate temperatures for annealing/extension of the particular primers used.

8. On or before April 15, 1991 a control PCR experiment was carried out without DNA. The control experiment further confirmed to me that the experiment worked.

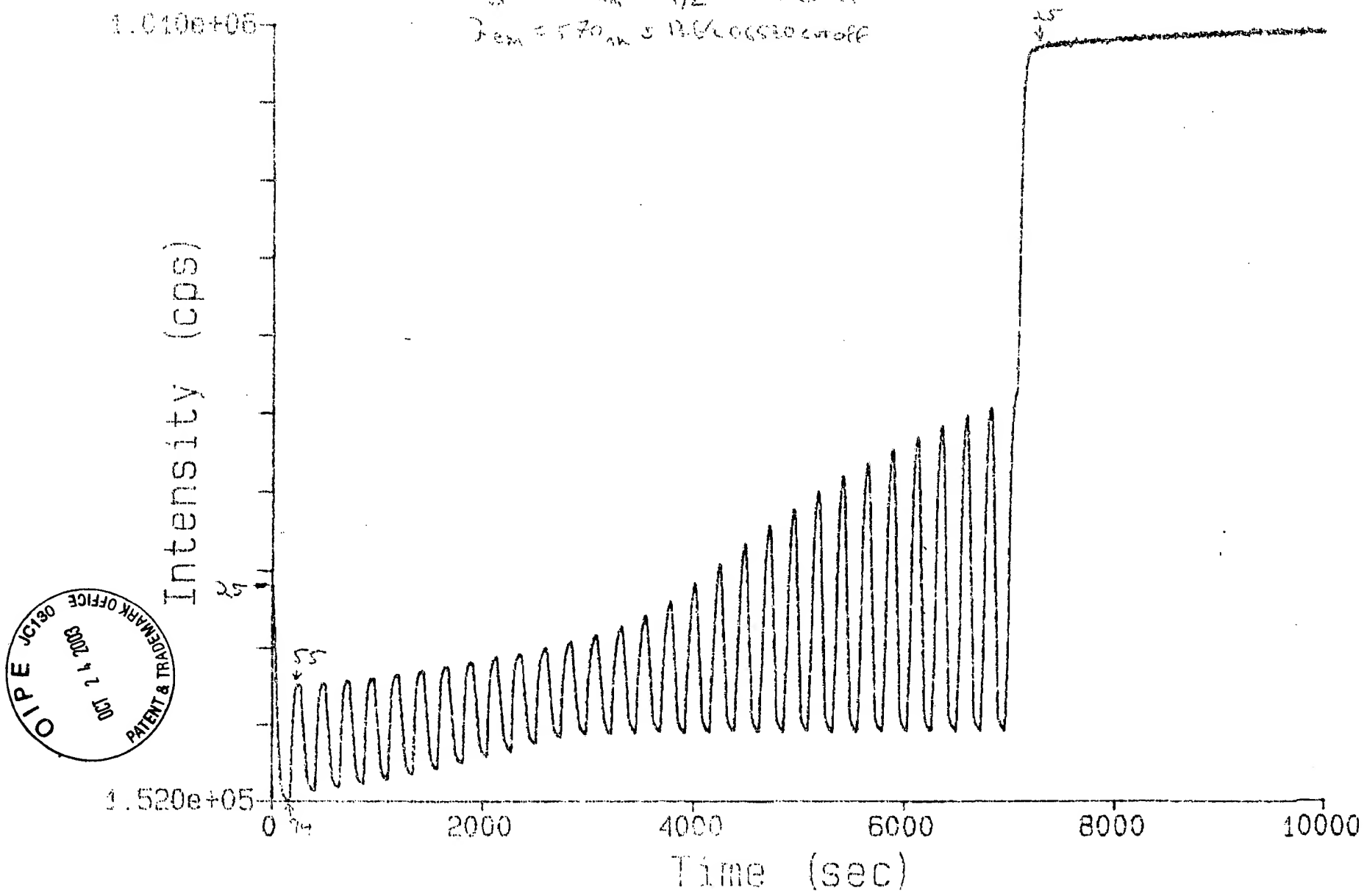
9. At some point after this experiment was performed, I prepared a manuscript that describes exactly the steps performed in the experiment and includes a fluorescence trace as Figure 5 of a subsequent run of the exact same experiment. I submitted this article to the scientific journal *BioTechnology* for publication. A copy of the subsequently published article is, Higuchi *et al.*, 1992, *BioTechnology* 10:413-417, is attached hereto as EXHIBIT B.

10. I declare further that all statements made herein of my own knowledge are true, that I believe that any statements herein made on information and belief are true, and that I acknowledge that any willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and may jeopardize the validity of the application or any patent issuing thereon.

Date: October 6, 2003

Russell G. Higuchi, Ph.D.

CMO
RMS Law Dept.



RESEARCH

SIMULTANEOUS AMPLIFICATION AND DETECTION OF SPECIFIC DNA SEQUENCES

Russell Higuchi*, Gavin Dollinger[1], P. Sean Walsh and Robert Griffith

Roche Molecular Systems, Inc., 1400 53rd St., Emeryville, CA 94608. [1]Chiron Corporation, 1400 53rd St., Emeryville, CA 94608. *Corresponding author.

We have enhanced the polymerase chain reaction (PCR) such that specific DNA sequences can be detected without opening the reaction tube. This enhancement requires the addition of ethidium bromide (EtBr) to a PCR. Since the fluorescence of EtBr increases in the presence of double-stranded (ds) DNA an increase in fluorescence in such a PCR indicates a positive amplification, which can be easily monitored externally. In fact, amplification can be continuously monitored in order to follow its progress. The ability to simultaneously amplify specific DNA sequences and detect the product of the amplification both simplifies and improves PCR and may facilitate its automation and more widespread use in the clinic or in other situations requiring high sample throughput.

Although the potential benefits of PCR[1] to clinical diagnostics are well known[2,3], it is still not widely used in this setting, even though it is four years since thermostable DNA polymerases[4] made PCR practical. Some of the reasons for its slow acceptance are high cost, lack of automation of pre- and post-PCR processing steps, and false positive results from carryover-contamination. The first two points are related in that labor is the largest contributor to cost at the present stage of PCR development. Most current assays require some form of "downstream" processing once thermocycling is done in order to determine whether the target DNA sequence was present and has amplified. These include DNA hybridization[5,6], gel electrophoresis with or without use of restriction digestion[7,8], HPLC[9], or capillary electrophoresis[10]. These methods are labor-intense, have low throughput, and are difficult to automate. The third point is also closely related to downstream processing. The handling of the PCR product in these downstream processes increases the chances that amplified DNA will spread through the typing lab, resulting in a risk of "carryover" false positives in subsequent testing[11].

These downstream processing steps would be eliminated if specific amplification and detection of amplified DNA took place simultaneously within an unopened reaction vessel. Assays in which such different processes take place without the need to separate reaction components have been termed "homogeneous". No truly homogeneous PCR assay has been demonstrated to date, although progress towards this end has been reported. Chehab, et al.[12], developed a PCR product detection scheme using fluorescent primers that resulted in a fluorescent PCR product. Allele-specific primers, each with different fluorescent tags, were used to indicate the genotype of the DNA. However, the unincorporated primers must still be removed in a downstream process in order to visualize the result. Recently, Holland, et al.[13], developed an assay in which the endogenous 5′ exonuclease assay of *Taq* DNA polymerase was exploited to cleave a labeled oligonucleotide probe. The probe would only cleave if PCR amplification had produced its complementary sequence. In order to detect the cleavage products, however, a subsequent process is again needed.

We have developed a truly homogeneous assay for PCR and PCR product detection based upon the greatly increased fluorescence that ethidium bromide and other DNA binding dyes exhibit when they are bound to ds-DNA[14–16]. As outlined in Figure 1, a prototypic PCR

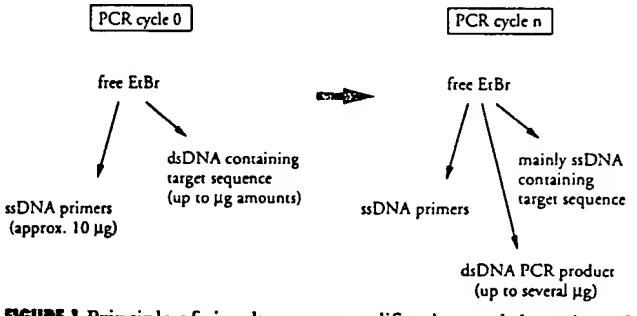


FIGURE 1 Principle of simultaneous amplification and detection of PCR product. The components of a PCR containing EtBr that are fluorescent are listed—EtBr itself, EtBr bound to either ssDNA or dsDNA. There is a large fluorescence enhancement when EtBr is bound to DNA and binding is greatly enhanced when DNA is double-stranded. After sufficient (n) cycles of PCR, the net increase in dsDNA results in additional EtBr binding, and a net increase in total fluorescence.

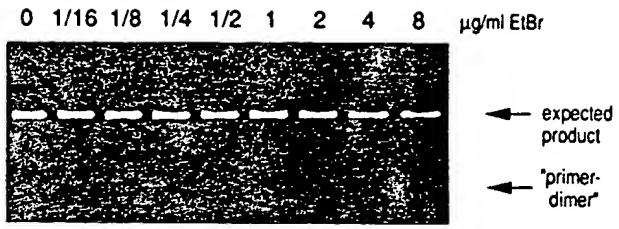


FIGURE 2 Gel electrophoresis of PCR amplification products of the human, nuclear gene, HLA DQα, made in the presence of increasing amounts of EtBr (up to 8 µg/ml). The presence of EtBr has no obvious effect on the yield or specificity of amplification.

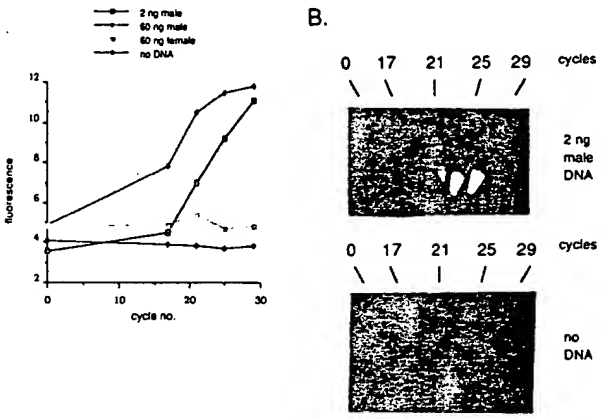


FIGURE 3 (A) Fluorescence measurements from PCRs that contain 0.5 µg/ml EtBr and that are specific for Y-chromosome repeat sequences. Five replicate PCRs were begun containing each of the DNAs specified. At each indicated cycle, one of the five replicate PCRs for each DNA was removed from thermocycling and its fluorescence measured. Units of fluorescence are arbitrary. (B) UV photography of PCR tubes (0.5 ml Eppendorf-style, polypropylene micro-centrifuge tubes) containing reactions, those starting from 2 ng male DNA and control reactions without any DNA, from (A).

begins with primers that are single-stranded DNA (ssDNA), dNTPs, and DNA polymerase. An amount of dsDNA containing the target sequence (target DNA) is also typically present. This amount can vary, depending on the application, from single-cell amounts of DNA[17] to micrograms per PCR[18]. If EtBr is present, the reagents that will fluoresce, in order of increasing fluorescence, are free EtBr itself, and EtBr bound to the single-stranded DNA primers and to the double-stranded target DNA (by its intercalation between the stacked bases of the DNA double-helix). After the first denaturation cycle, target DNA will be largely single-stranded. After a PCR is completed, the most significant change is the increase in the amount of dsDNA (the PCR product itself) of up to several micrograms. Formerly free EtBr is bound to the additional dsDNA, resulting in an increase in fluorescence. There is also some decrease in the amount of ssDNA primer, but because the binding of EtBr to ssDNA is much less than to dsDNA, the effect of this change on the total fluorescence of the sample is small. The fluorescence increase can be measured by directing excitation illumination through the walls of the amplification vessel before and after, or even continuously during, thermocycling.

RESULTS

PCR in the presence of EtBr. In order to assess the affect of EtBr in PCR, amplifications of the human HLA DQα gene[19] were performed with the dye present at concentrations from 0.06 to 8.0 µg/ml (a typical concentration of EtBr used in staining of nucleic acids following gel electrophoresis is 0.5 µg/ml). As shown in Figure 2, gel electrophoresis revealed little or no difference in the yield or quality of the amplification product whether EtBr was absent or present at any of these concentrations, indicating that EtBr does not inhibit PCR.

Detection of human Y-chromosome specific sequences. Sequence-specific, fluorescence enhancement of EtBr as a result of PCR was demonstrated in a series of amplifications containing 0.5 µg/ml EtBr and primers specific to repeat DNA sequences found on the human Y-chromosome[20]. These PCRs initially contained either 60 ng male, 60 ng female, 2 ng male human or no DNA. Five replicate PCRs were begun for each DNA. After 0, 17, 21, 24 and 29 cycles of thermocycling, a PCR for each DNA was removed from the thermocycler, and its fluorescence measured in a spectrofluorometer and plotted vs. amplification cycle number (Fig. 3A). The shape of this curve reflects the fact that by the time an increase in fluorescence can be detected, the increase in DNA is becoming linear and not exponential with cycle number. As shown, the fluorescence increased about three-fold over the background fluorescence for the PCRs containing human male DNA, but did not significantly increase for negative control PCRs, which contained either no DNA or human female DNA. The more male DNA present to begin with—60 ng versus 2 ng—the fewer cycles were needed to give a detectable increase in fluorescence. Gel electrophoresis on the products of these amplifications showed that DNA fragments of the expected size were made in the male DNA containing reactions and that little DNA synthesis took place in the control samples.

In addition, the increase in fluorescence was visualized by simply laying the completed, unopened PCRs on a UV transilluminator and photographing them through a red filter. This is shown in figure 3B for the reactions that began with 2 ng male DNA and those with no DNA.

Detection of specific alleles of the human β-globin gene. In order to demonstrate that this approach has adequate specificity to allow genetic screening, a detection of the sickle-cell anemia mutation was performed. Figure 4 shows the fluorescence from completed amplifications containing EtBr (0.5 µg/ml) as detected by photography of the reaction tubes on a UV transilluminator. These reactions were performed using primers specific for either the wild-type or sickle-cell mutation of the human β-globin gene[21]. The specificity for each allele is imparted by placing the sickle-mutation site at the terminal 3′ nucleotide of one primer. By using an appropriate primer annealing temperature, primer extension—and thus amplification—can take place only if the 3′ nucleotide of the primer is complementary to the β-globin allele present[21,22].

Each pair of amplifications shown in Figure 4 consists of a reaction with either the wild-type allele specific (left tube) or sickle-allele specific (right tube) primers. Three different DNAs were typed: DNA from a homozygous, wild-type β-globin individual (AA); from a heterozygous sickle β-globin individual (AS); and from a homozygous sickle β-globin individual (SS). Each DNA (50 ng genomic DNA to start each PCR) was analyzed in triplicate (3 pairs

of reactions each). The DNA type was reflected in the relative fluorescence intensities in each pair of completed amplifications. There was a significant increase in fluorescence only where a β-globin allele DNA matched the primer set. When measured on a spectrofluorometer (data not shown), this fluorescence was about three times that present in a PCR where both β-globin alleles were mismatched to the primer set. Gel electrophoresis (not shown) established that this increase in fluorescence was due to the synthesis of nearly a microgram of a DNA fragment of the expected size for β-globin. There was little synthesis of dsDNA in reactions in which the allele-specific primer was mismatched to both alleles.

Continuous monitoring of a PCR. Using a fiber optic device, it is possible to direct excitation illumination from a spectrofluorometer to a PCR undergoing thermocycling and to return its fluorescence to the spectrofluorometer. The fluorescence readout of such an arrangement, directed at an EtBr-containing amplification of Y-chromosome specific sequences from 25 ng of human male DNA, is shown in Figure 5. The readout from a control PCR with no target DNA is also shown. Thirty cycles of PCR were monitored for each.

The fluorescence trace as a function of time clearly shows the effect of the thermocycling. Fluorescence intensity rises and falls inversely with temperature. The fluorescence intensity is minimum at the denaturation temperature (94°C) and maximum at the annealing/extension temperature (50°C). In the negative-control PCR, these fluorescence maxima and minima do not change significantly over the thirty thermocycles, indicating that there is little dsDNA synthesis without the appropriate target DNA, and there is little if any bleaching of EtBr during the continuous illumination of the sample.

In the PCR containing male DNA, the fluorescence maxima at the annealing/extension temperature begin to increase at about 4000 seconds of thermocycling, and continue to increase with time, indicating that dsDNA is being produced at a detectable level. Note that the fluorescence minima at the denaturation temperature do not significantly increase, presumably because at this temperature there is no dsDNA for EtBr to bind. Thus the course of the amplification is followed by tracking the fluorescence increase at the annealing temperature. Analysis of the products of these two amplifications by gel electrophoresis showed a DNA fragment of the expected size for the male DNA containing sample and no detectable DNA synthesis for the control sample.

DISCUSSION

Downstream processes such as hybridization to a sequence-specific probe can enhance the specificity of DNA detection by PCR. The elimination of these processes means that the specificity of this homogeneous assay depends solely on that of PCR. In the case of sickle-cell disease, we have shown that PCR alone has sufficient DNA sequence specificity to permit genetic screening. Using appropriate amplification conditions, there is little non-specific production of dsDNA in the absence of the appropriate target allele.

The specificity required to detect pathogens can be more or less than that required to do genetic screening, depending on the number of pathogens in the sample and the amount of other DNA that must be taken with the sample. A difficult target is HIV, which requires detection of a viral genome that can be at the level of a few copies per thousands of host cells[6]. Compared with genetic screening, which is performed on cells containing at least one copy of the target sequence, HIV detection requires both more specificity and the input of more total

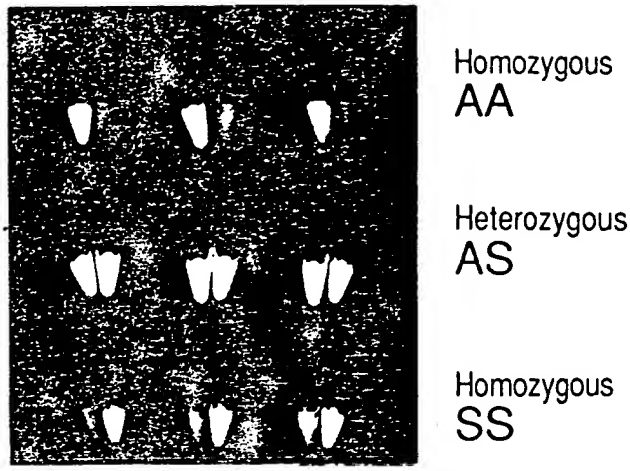


FIGURE 4 UV photography of PCR tubes containing amplifications using EtBr that are specific to wild-type (A) or sickle (S) alleles of the human β-globin gene. The left of each pair of tubes contains allele-specific primers to the wild-type alleles, the right tube primers to the sickle allele. The photograph was taken after 30 cycles of PCR, and the input DNAs and the alleles they contain are indicated. Fifty ng of DNA was used to begin PCR. Typing was done in triplicate (3 pairs of PCRs) for each input DNA.

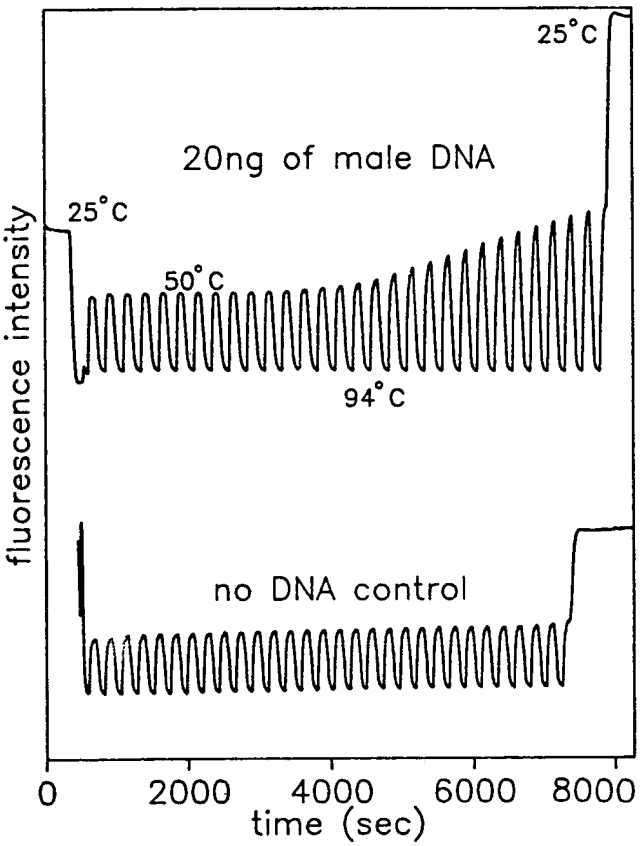


FIGURE 5 Continuous, real-time monitoring of a PCR. A fiber optic was used to carry excitation light to a PCR in progress and also emitted light back to a fluorometer (see Experimental Protocol). Amplification using human male-DNA specific primers in a PCR starting with 20 ng of human male DNA (top), or in a control PCR without DNA (bottom), were monitored. Thirty cycles of PCR were followed for each. The temperature cycled between 94°C (denaturation) and 50°C (annealing and extension). Note in the male DNA PCR, the cycle (time) dependent increase in fluorescence at the annealing/extension temperature.

DNA—up to microgram amounts—in order to have sufficient numbers of target sequences. This large amount of starting DNA in an amplification significantly increases the background fluorescence over which any additional fluorescence produced by PCR must be detected. An additional complication that occurs with targets in low copy-number is the formation of the "primer-dimer" artifact. This is the result of the extension of one primer using the other primer as a template. Although this occurs infrequently, once it occurs the extension product is a substrate for PCR amplification, and can compete with true PCR targets if those targets are rare. The primer-dimer product is of course dsDNA and thus is a potential source of false signal in this homogeneous assay.

To increase PCR specificity and reduce the effect of primer-dimer amplification, we are investigating a number of approaches, including the use of nested-primer amplifications that take place in a single tube[3], and the "hot-start", in which nonspecific amplification is reduced by raising the temperature of the reaction before DNA synthesis begins[23]. Preliminary results using these approaches suggest that primer-dimer is effectively reduced and it is possible to detect the increase in EtBr fluorescence in a PCR instigated by a single HIV genome in a background of 10^5 cells. With larger numbers of cells, the background fluorescence contributed by genomic DNA becomes problematic. To reduce this background, it may be possible to use sequence-specific DNA-binding dyes that can be made to preferentially bind PCR product over genomic DNA by incorporating the dye-binding DNA sequence into the PCR product through a 5′ "add-on" to the oligonucleotide primer[24].

We have shown that the detection of fluorescence generated by an EtBr-containing PCR is straightforward, both once PCR is completed and continuously during thermocycling. The ease with which automation of specific DNA detection can be accomplished is the most promising aspect of this assay. The fluorescence analysis of completed PCRs is already possible with existing instrumentation in 96-well format[25]. In this format, the fluorescence in each PCR can be quantitated before, after, and even at selected points during thermocycling by moving the rack of PCRs to a 96-microwell plate fluorescence reader[26].

The instrumentation necessary to continuously monitor multiple PCRs simultaneously is also simple in principle. A direct extension of the apparatus used here is to have multiple fiberoptics transmit the excitation light and fluorescent emissions to and from multiple PCRs. The ability to monitor multiple PCRs continuously may allow quantitation of target DNA copy number. Figure 3 shows that the larger the amount of starting target DNA, the sooner during PCR a fluorescence increase is detected. Preliminary experiments (Higuchi and Dollinger, manuscript in preparation) with continuous monitoring have shown a sensitivity to two-fold differences in initial target DNA concentration.

Conversely, if the number of target molecules is known—as it can be in genetic screening—continuous monitoring may provide a means of detecting false positive and false negative results. With a known number of target molecules, a true positive would exhibit detectable fluorescence by a predictable number of cycles of PCR. Increases in fluorescence detected before or after that cycle would indicate potential artifacts. False negative results due to, for example, inhibition of DNA polymerase, may be detected by including within each PCR an inefficiently amplifying marker. This marker results in a fluorescence increase only after a large number of cycles—many more than are necessary to detect a true positive. If a sample fails to have a fluorescence increase after this many cycles, inhibition may be suspected. Since, in this assay, conclusions are drawn based on the presence or absence of fluorescence signal alone, such controls may be important. In any event, before any test based on this principle is ready for the clinic, an assessment of its false positive/false negative rates will need to be obtained using a large number of known samples.

In summary, the inclusion in PCR of dyes whose fluorescence is enhanced upon binding dsDNA makes it possible to detect specific DNA amplification from outside the PCR tube. In the future, instruments based upon this principle may facilitate the more widespread use of PCR in applications that demand the high throughput of samples.

EXPERIMENTAL PROTOCOL

Human HLA-DQα gene amplifications containing EtBr. PCRs were set up in 100 µl volumes containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 4 mM $MgCl_2$; 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT); 20 pmole each of human HLA-DQα gene specific oligonucleotide primers GH26 and GH27[19] and approximately 10^7 copies of DQα PCR product diluted from a previous reaction. Ethidium bromide (EtBr; Sigma) was used at the concentrations indicated in Figure 2. Thermocycling proceeded for 20 cycles in a model 480 thermocycler (Perkin-Elmer Cetus, Norwalk, CT) using a "step-cycle" program of 94°C for 1 min. denaturation and 60°C for 30 sec. annealing and 72°C for 30 sec. extension.

Y-chromosome specific PCR. PCRs (100 µl total reaction volume) containing 0.5 µg/ml EtBr were prepared as described for HLA-DQα, except with different primers and target DNAs. These PCRs contained 15 pmole each male DNA-specific primers Y1.1 and Y1.2[20], and either 60 ng male, 60 ng female, 2 ng male, or no human DNA. Thermocycling was 94°C for 1 min. and 60°C for 1 min using a "step-cycle" program. The number of cycles for a sample were as indicated in Figure 3. Fluorescence measurement is described below.

Allele-specific, human β-globin gene PCR. Amplifications of 100 µl volume using 0.5 µg/ml of EtBr were prepared as described for HLA-DQα above except with different primers and target DNAs. These PCRs contained either primer pair HGP2/HβI4A (wild-type globin specific primers) or HGP2/HβI4S (sickle-globin specific primers) at 10 pmole each primer per PCR. These primers were developed by Wu et al.[21]. Three different target DNAs were used in separate amplifications—50 ng each of human DNA that was homozygous for the sickle trait (SS), DNA that was heterozygous for the sickle trait (AS), or DNA that was homozygous for the w.t. globin (AA). Thermocycling was for 30 cycles at 94°C for 1 min. and 55°C for 1 min. using a "step-cycle" program. An annealing temperature of 55°C had been shown by Wu et al.[21] to provide allele-specific amplification. Completed PCRs were photographed through a red filter (Wratten 23A) after placing the reaction tubes atop a model TM-36 transilluminator (UV-products San Gabriel, CA).

Fluorescence measurement. Fluorescence measurements were made on PCRs containing EtBr in a Fluorolog-2 fluorometer (SPEX, Edison, NJ). Excitation was at the 500 nm band with about 2 nm bandwidth with a GG 435 nm cut-off filter (Melles Grist, Inc., Irvine, CA) to exclude second-order light. Emitted light was detected at 570 nm with a bandwidth of about 7 nm. An OG 530 nm cut-off filter was used to remove the excitation light.

Continuous fluorescence monitoring of PCR. Continuous monitoring of a PCR in progress was accomplished using the spectrofluorometer and settings described above as well as a fiberoptic accessory (SPEX cat. no. 1950) to both send excitation light to, and receive emitted light from, a PCR placed in a well of a model 480 thermocycler (Perkin-Elmer Cetus). The probe end of the fiberoptic cable was attached with "5 minute-epoxy" to the open top of a PCR tube (a 0.5 ml polypropylene centrifuge tube with its cap removed) effectively sealing it. The exposed top of the PCR tube and the end of the fiberoptic cable were shielded from room light and the room lights were kept dimmed during each run. The monitored PCR was an amplification of Y-chromosome-specific repeat sequences as described above, except using an annealing/extension temperature of 50°C. The reaction was covered with mineral oil (2 drops) to prevent evaporation. Thermocycling and fluorescence measurement were started simultaneously. A time-base scan with a 10 second integration time

was used and the emission signal was ratioed to the excitation signal to control for changes in light-source intensity. Data were collected using the dm3000f, version 2.5 (SPEX) data system.

Acknowledgments

We thank Bob Jones for help with the spectrofluormetric measurements and Heatherbell Fong for editing this manuscript.

References

1. Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. 1986. Specific enzymatic amplification of DNA *in vitro:* The polymerase chain reaction. CSHSQB **51**:263–273.
2. White, T. J., Arnheim, N. and Erlich, H. A. 1989. The polymerase chain reaction. Trends Genet. **5**:185–189.
3. Erlich, H. A., Gelfand, D. and Sninsky, J. J. 1991. Recent advances in the polymerase chain reaction. Science **252**:1643–1651.
4. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
5. Saiki, R. K., Walsh, P. S., Levenson, C. H. and Erlich, H. A. 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Natl. Acad. Sci. USA **86**:6230–6234.
6. Kwok, S. Y., Mack, D. H., Mullis, K. B., Poiesz, B. J., Ehrlich, G. D., Blair, D. and Friedman-Kien, A. S. 1987. Identification of human immunodeficiency virus sequences by using *in vitro* enzymatic amplification and oligomer cleavage detection. J. Virol. **61**:1690–1694.
7. Chehab, F. F., Doherty, M., Cai, S. P., Kan, Y. W., Cooper, S. and Rubin, E. M. 1987. Detection of sickle cell anemia and thalassemias. Nature **329**:293–294.
8. Horn, G. T., Richards, B. and Klinger, K. W. 1989. Amplification of a highly polymorphic VNTR segment by the polymerase chain reaction. Nuc. Acids Res. **16**:2140.
9. Katz, E. D. and Dong, M. W. 1990. Rapid analysis and purification of polymerase chain reaction products by high-performance liquid chromatography. Biotechniques **8**:546–555.
10. Heiger, D. N., Cohen, A. S. and Karger, B. L. 1990. Separation of DNA restriction fragments by high performance capillary electrophoresis with low and zero crosslinked polyacrylamide using continuous and pulsed electric fields. J. Chromatogr. **516**:33–48.
11. Kwok, S. Y. and Higuchi, R. G. 1989. Avoiding false positives with PCR. Nature **339**:237–238.
12. Chehab, F. F. and Kan, Y. W. 1989. Detection of specific DNA sequences by fluorescence amplification: a color complementation assay. Proc. Natl. Acad. Sci. USA **86**:9178–9182.
13. Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonulease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA **88**:7276–7280.
14. Markovits, J., Roques, B. P. and Le Pecq, J. B. 1979. Ethidium dimer: a new reagent for the fluorimetric determination of nucleic acids. Anal. Biochem. **94**:259–264.
15. Kapuscinski, J. and Szer, W. 1979. Interactions of 4′,6-diamidine-2-phenylindole with synthetic polynucleotides. Nuc. Acids Res. **6**:3519–3534.
16. Searle, M. S. and Embrey, K. J. 1990. Sequence-specific interaction of Hoescht 33258 with the minor groove of an adenine-tract DNA duplex studied in solution by ^{1}H NMR spectroscopy. Nuc. Acids Res. **18**:3753–3762.
17. Li, H. H., Gyllensten, U. B., Cui, X. F., Saiki, R. K., Erlich, H. A. and Arnheim, N. 1988. Amplification and analysis of DNA sequences in single human sperm and diploid cells. Nature **335**:414–417.
18. Abbott, M. A., Poiexz, B. J., Byrne, B. C., Kwok, S. Y., Sninsky, J. J. and Erlich, H. A. 1988. Enzymatic gene amplification: qualitative and quantitative methods for detecting proviral DNA amplified *in vitro*. J. Infect. Dis. **158**:1158.
19. Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1986. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature **324**:163–166.
20. Kogan, S. C., Doherty, M. and Gitschier, J. 1987. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. N. Engl. J. Med. **317**:985–990.
21. Wu, D. Y., Ugozzoli, L., Pal, B. K. and Wallace, R. B. 1989. Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc. Natl. Acad. Sci. USA **86**:2757–2760.
22. Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C. and Sninsky, J. J. 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. Nuc. Acids Res. **18**:999–1005.
23. Chou, Q., Russell, M., Birch, D., Raymond, J. and Bloch, W. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Submitted.
24. Higuchi, R. 1989. Using PCR to engineer DNA, p. 61–70. *In:* PCR Technology. H. A. Erlich (Ed.). Stockton Press, New York, N.Y.
25. Haff, L., Atwood, J. G., DiCesare, J., Katz, E., Picozza, E., Williams, J. F. and Woudenberg, T. 1991. A high-performance system for automation of the polymerase chain reaction. Biotechniques **10**:102–103, 106–112.
26. Tumosa, N. and Kahan, L. 1989. Fluorescent EIA screening of monoclonal antibodies to cell surface antigens. J. Immun. Meth. **116**:59–63.